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was intended since it is well known in the art that the ileal loop assay is done in the rabbit and not in the mouse. See e.g., Giannelli et al., "Protease Susceptibility and Toxicity of Heat-Labile Enterotoxins with a Mutation in the Active Site or in the Protease-Sensitive Loop," *Infection and Immunity* 65(1):331-334, included herewith.

Thus, no new matter is being added by the foregoing amendment and entry thereof is respectfully requested.

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Protease Susceptibility and Toxicity of Heat-Labile Enterotoxins with a Mutation in the Active Site or in the Protease-Sensitive Loop

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To generate nontoxic derivatives of *Escherichia coli* heat-labile enterotoxin (LT), site-directed mutagenesis has been used to change either the amino acid residues located in the catalytic site (M. Pizza, M. Domenighini, W. Hol, V. Giannelli, M. R. Fontana, M. M. Giuliani, C. Magagnoli, S. Peppoloni, R. Manetti, and R. Rappuoli, Mol. Microbiol. 14:51–60, 1994) or those located in the proteolytically sensitive loop that joins the A1 and A2 moieties of the A subunit (C. C. R. Grant, R. J. Messer, and W. J. Cieplack, Infect. Immun. 62:4270–4278, 1994; B. L. Dickinson and J. D. Clements, Infect. Immun. 63:1617–1623, 1995). In this work, we compared the in vitro and in vivo toxic properties and the resistance to protease digestion of the prototype molecules obtained by both approaches (LT-K63 and LT-R192G, respectively). As expected, LT-K63 was normally processed by proteases, while LT-R192G showed increased resistance to trypsin in vitro and was digested by trypsin only under denaturing conditions (3.5 M urea) or by intestinal proteases. No toxicity was detected with the LT-K63 mutant, even when 40 μg and 1 mg were used in the in vitro and in vivo assays, respectively. In marked contrast, LT-R192G showed only a modest (10-fold) reduction in toxicity in Y1 cells with a delay in the appearance of the toxic activity and had toxicity comparable to that of wild-type LT in the rabbit ileal loop assay. We conclude that mutagenesis of the active site generates molecules that are fully devoid of toxicity, while mutagenesis of the A1-A2 loop generates molecules that are resistant to trypsin in vitro but still susceptible to proteolytic activation by proteases other than trypsin, and therefore they may still be toxic in tissue culture and in vivo.

Escherichia coli heat-labile enterotoxin (LT) (1, 2) belongs the family of bacterial ADP-ribosylating toxins (11). Like all the toxins of this family, such as diphtheria, cholera, and pertussis toxins and *Pseudomonas* exotoxin A, LT is composed of two distinct domains: the enzymatically active A subunit that is responsible for the toxicity and the B subunit that binds the receptor and facilitates the entry of the A domain into eukaryotic cells. The B subunit is composed of five identical monomers of 11.8 kDa each that assemble into a pentameric ringlike structure, which is able to bind the GM_1 receptor on the surface of eukaryotic cells (14), and a central cavity that houses the carboxy-terminal portion of the A subunit.

The A subunit is composed of two domains, A1 and A2, of 21.8 and 5.4 kDa, respectively, that derive from proteolytic processing of the A subunit and are held together by a disulfide bridge. The A1 domain has a globular structure and contains the catalytic site of an enzyme that modifies Gs_α and other GTP-binding proteins, causing an increase of intracellular cyclic AMP, prostaglandin production, and stimulation of the intestinal gut sensory neurons. The combination of these activities results in intestinal accumulation of fluids and the consequent watery diarrhea characteristic of the disease induced by LT. The A2 domain is made mostly by a long alpha-helix connecting the A1 domain to the B subunit (12, 13). Activation of the enzymatic activity requires proteolytic cleavage of the ^{187}CGNSSRTITGDTC^{199} loop and reduction of the disulfide bond linking Cys-187 to Cys-199, connecting the A1 and A2 domains.

LT is a powerful immunogen and a potent mucosal adjuvant; however, the toxicity of this molecule has precluded its development for human use. Therefore, several attempts have been made to mutagenize the gene and obtain nontoxic derivatives useful as adjuvants (4, 5) or immunogens (9, 10). The two molecules that have been reported as more promising differ in their design and properties: LT-K63 contains a Ser-63→Lys mutation in the active site that makes the molecule enzymatically inactive (8, 9), and LT-R192G contains an Arg-192→Gly mutation in the loop that makes it resistant to trypsin digestion (4, 7). Although both molecules have been well described in the literature, different methods were used for their evaluation in the original studies and in some cases partially conflicting findings on their toxicity were reported (4, 7). Grant et al. showed that trypsin-treated LT-R192G induced a cytotonic activity on CHO cells similar to that induced by the untreated wild-type toxin and exhibited a delay in the ability to increase intracellular levels of cyclic AMP in Caco-2 cells (7). Dickinson and Clements showed that LT-R192G was 1,000 times less toxic than wild-type LT for Y1 cells (4). In this investigation, we analyzed and compared, in parallel studies, the in vitro and in vivo properties of LT-K63, LT-R192G, and wild-type LT.

The mutant molecules were obtained by site-directed mutagenesis as previously described (6, 8) and purified from the periplasm of *E. coli* strains (8). To test their protease susceptibility, purified wild-type LT, LT-K63, and LT-R192G were treated with trypsin (1:100 trypsin/protein ratio) at 37°C and samples were collected at different times and analyzed by

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Western blotting (15). As shown in Fig. 1A, the trypsin treatment caused partial nicking of the A subunit of wild-type LT and LT-K63 after 5 min of incubation and complete nicking after 90 min. The A subunit of LT-R192G was undigested, even after 90 min of incubation at 37°C, confirming that this mutant is resistant to trypsin treatment. To further characterize the influence of the mutation on resistance to trypsin cleavage, the same experiment was performed on partially unfolded molecules under denaturing conditions (3.5 M urea). In this experiment (Fig. 1B), the A subunit of the LT-R192G mutant was cleaved by trypsin after 5 min of incubation, generating a new fragment migrating more slowly than the A1 fragment deriving from LT and LT-K63. After 15 min of incubation, the electrophoretic profiles of the three molecules appeared different. Wild-type LT and LT-K63 generated a number of different fragments, of which the main one was still represented by A1. In the case of LT-R192G, two fragments different in mobility were generated, one migrating slower and the other faster than the A1 fragment. After 60 min of incubation, the A subunits of LT and LT-K63 were completely degraded, whereas some of the A subunit of LT-R192G was still undigested. In conclusion, LT-R192G appears to be fully resistant to trypsin treatment under physiological conditions and more resistant than LT and LT-K63 under denaturing conditions.

To test whether the LT-R192G mutant would be resistant to activation by proteases other than trypsin and likely to be found in the extracellular compartments in vivo, we used the intestinal wash of two mice as a pool of proteases. Incubation of wild-type LT and LT-R192G in the presence of these proteases at 37°C showed that the A subunits of LT and LT-R192G were both partially cleaved after 30 min (Fig. 1C). However, while LT generated mostly the A1 fragment, LT-R192G was digested more slowly and generated a different pattern of fragmentation. After 4 h of incubation, the A subunits of both LT and LT-R192G were completely digested by the proteases. These results suggest that the A subunit of LT-R192G, although more resistant than LT to protease attack, may be activated, in vivo, by proteases other than trypsin that generate alternative proteolytic fragments.

Finally, we tested the in vitro toxicity of the mutant molecules in tissue culture cells, where discordant results had been reported for LT-R192G. In fact, Grant et al. had reported a toxicity similar to that of wild-type LT in CHO cells (7), while Dickinson and Clements had reported 1,000-fold-reduced toxicity compared with LT in Y1 cells (4). We used Y1 cells, which are more sensitive than CHO cells. Twofold dilutions of LT, LT-R192G, and LT-K63 were added to wells containing 5×10^4 cells, and the appearance of the morphological changes showing cell toxicity was monitored every hour for 27 h. As shown in Fig. 2, toxicity was time and dose dependent. The toxicity of 50 ng of wild-type LT was detected as early as 3 h after toxin addition. Two hundred picograms was toxic after 4 h, whereas 25 pg required 7 h. After 24 h, even 3 pg of toxin was toxic. In marked contrast, LT-R192G did not show typical toxicity for 8 h, even when a very large amount of LT-R192G (100 ng) was used. During this period, however, the Y1 cells showed a morphological phenotype that was different from that of the control (Fig. 3B and D, respectively), suggesting initial but not full toxicity. Finally, starting 10 h after the addition of LT-R192G, the cells began to show full toxicity (Fig. 3F), even with 100 pg of the molecule. After 24 h, even 25 pg of LT-R192G was fully toxic, indicating that in this assay, after 24 h of incubation, LT-R192G is approximately 10 times less toxic than wild-type LT. LT-K63 showed no sign of toxicity at any time, even when 40 μg was used in the assay. The above results suggest that whereas LT-K63 is fully inactivated by the

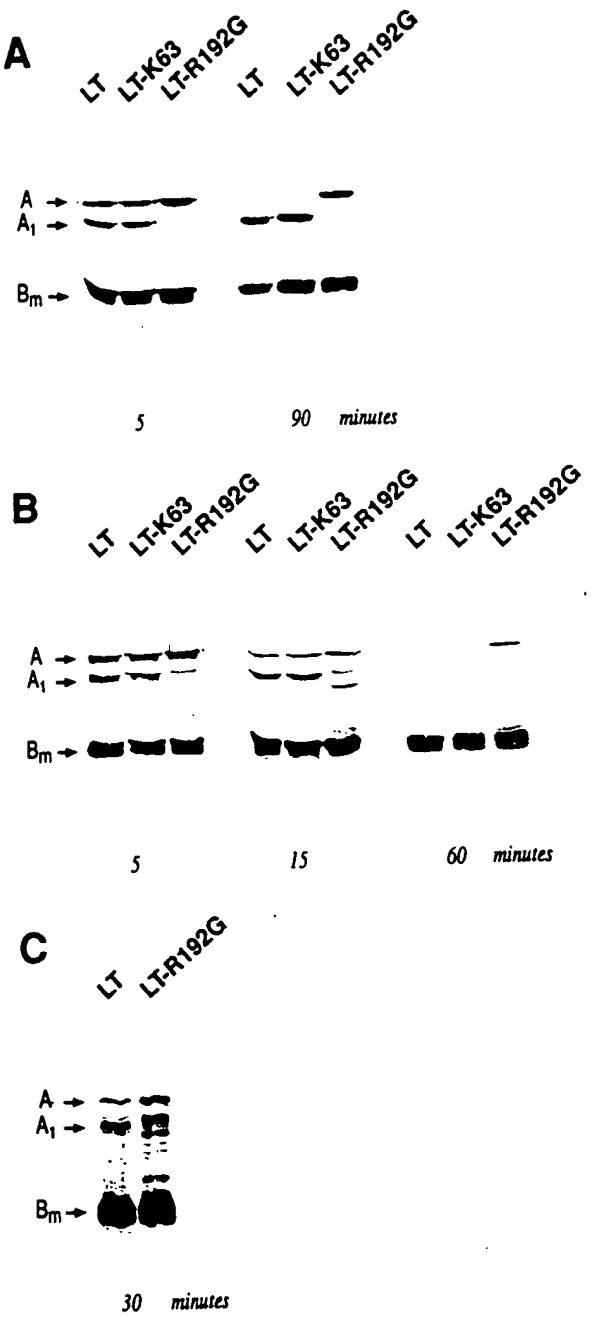

FIG. 1. Trypsin and intestinal protease sensitivity of wild-type LT and LT mutants. (A) Western blot analysis of LT molecules treated with trypsin (1:100 trypsin/protein ratio) after 5 and 90 min of incubation at 37°C. (B) Western blot analysis of LT molecules after treatment with trypsin (1:100 trypsin/protein ratio) in the presence of 3.5 M urea after 5, 15, and 60 min of incubation at 37°C. A 60-μg sample of LT or LT mutant protein was treated with 0.6 μg of trypsin. Each sample was analyzed by Western blotting with rabbit anti-LT polyclonal antibodies at a dilution of 1/300. (C) Western blot analysis of wild-type LT and LT-R192G treated with intestinal proteases after 30 min of incubation at 37°C. LT-K63 (not shown) gave a pattern of proteolysis similar to that of wild-type LT. The anti-LT antibodies used did not show any cross-reaction with the intestinal proteases in control experiments (data not shown). The intestines of two mice were washed with 1 ml of PBS. A 20-μl volume of this solution was incubated with 7 μg of LT or LT-R192G at 37°C. The samples were analyzed by Western blotting with rabbit anti-LT polyclonal antibodies at a dilution of 1/350. B_m, B monomer.

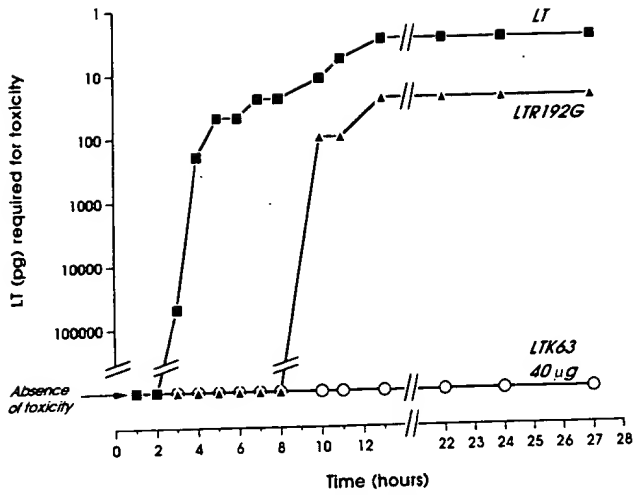

FIG. 2. Toxicity of wild-type LT and LT mutants in Y1 cells. Amounts of wild-type LT (■) or LT-R192G (▲) required to induce full toxicity in a Y1 cell assay during a time course experiment spanning 27 h are shown. When toxicity is not present, the data are plotted just above the *x* axis and identified by an arrow indicating the absence of toxicity. Only when toxicity is present are the data entered on the *y* axis. The maximum amounts tested in the assay were 100 ng of LT, 100 ng of LT-R192G, and 40 μg of LT-K63.

mutation in the active site, LT-R192G retains full toxicity but requires a longer time for activation. The above results also reconcile those reported in the literature. In fact, if one measured toxicity after 24 h, one would agree with Grant et al. that there is little difference between the toxic activities of LT and LT-R192G. However, if one measured toxicity after 4 to 8 h of incubation, one would agree with Dickinson and Clements that LT-R192G is 1,000-fold less toxic than wild-type LT.

Finally, we tested the mutant toxins in the rabbit ileal loop assay, which is believed to be the most reliable test to predict the in vivo toxicity of LT. The assay was performed as previously described (3), with different doses of the test molecules and two New Zealand rabbits per assay. More specifically, 12 to 14 loops, each 5 to 6 cm long, were made by starting at the end of the rabbit's small intestine and moving toward the stomach. One-milliliter samples of the toxins containing different amounts of protein were injected into ileal loops, and then the abdomen was closed. Six to eight loops were used for each mutant toxin, four loops were used for wild-type LT, and one loop was used for saline. The experiment was performed four times, each time in duplicate. After 18 to 20 h, the liquid accumulated in each loop was collected and measured with a syringe. The results of a representative experiment, reported in Table 1, show that 50 ng of wild-type LT or LT-R192G was able to induce fluid accumulation, whereas 10 ng did not. In marked contrast, even 1 mg of LTK63 was unable to induce fluid accumulation in the loop. These in vivo results confirm those previously obtained in vitro and show that mutations in the proteolytically sensitive loop may delay but not abolish toxicity, while mutations in the active site may produce fully nontoxic molecules. The observation that LT-R192G is still toxic in vitro and in vivo despite the in vitro resistance to trypsin attack may be explained by the fact that proteases other than trypsin are present in vivo and that these are still able to process and activate the mutant LT.

TABLE 1. Rabbit ileal loop assay results[a]

Dose (μg)	Fluid accumulation (ml/cm)		
	LT	LT-R192G	LT-K63
0.01	0.0	0.0	0.0
0.05	2.0	1.4	0.0
0.1	1.9	1.6	0.0
0.5	2.3	2.0	0.0
1	ND	1.9	0.0
5	ND	1.6	0.0
10	ND	1.8	0.0
50	ND	1.8	0.0
100	ND	ND	0.0
500	ND	ND	0.0
1,000	ND	ND	0.0

[a] Fluid accumulation induced by wild-type LT and mutant LTs is expressed as the ratio of the amount of fluid collected (in milliliters) to the length of each loop (in centimeters). ND, not done.

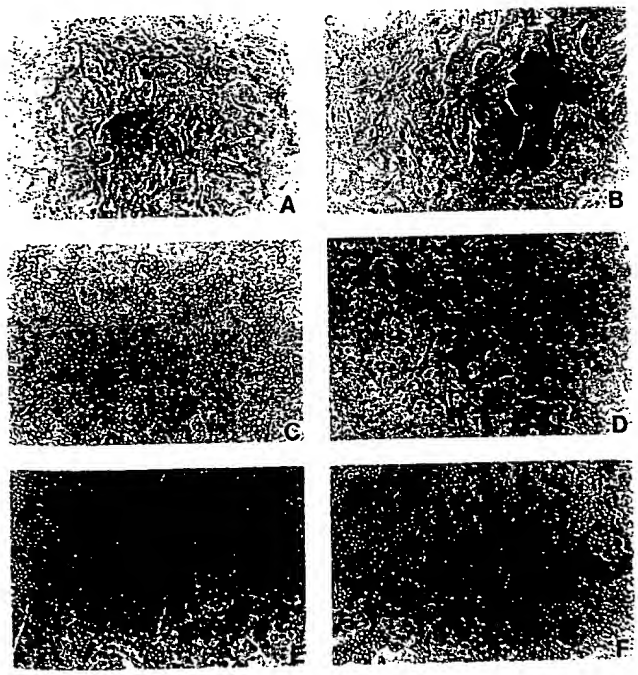

FIG. 3. Morphological changes induced by wild-type LT and LT-R192G in Y1 cells. Wells containing control Y1 cells (A and B) are compared with Y1 cells treated with 800 pg of wild-type LT or LT-R192G after 8 (C and D) and 24 (E and F) h of incubation at 37°C.

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